

Expression of α -farnesene synthase gene *AFS1* in relation to levels of α -farnesene and conjugated trienols in peel tissue of scald-susceptible ‘Law Rome’ and scald-resistant ‘Idared’ apple fruit

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Abstract

Nontreated control and 1-MCP-treated fruit of the scald-susceptible ‘Law Rome’ and scald-resistant ‘Idared’ cultivars were compared with respect to scald incidence, internal ethylene concentration (IEC), α -farnesene metabolism, and expression of the gene encoding α -farnesene synthase (*AFS1*), the final, rate-limiting enzyme in the α -farnesene biosynthetic pathway. The incidence of scald in nontreated ‘Law Rome’ apples after 20 weeks at 0.5 °C plus 1 week at 20 °C averaged 86%; 1-MCP treatment reduced increases in IEC and reduced the incidence of scald to less than 1%. Fruit of ‘Idared’ showed no scald regardless of the treatment. 1-MCP also inhibited α -farnesene production, suggesting that ethylene induces transcription of key genes involved in α -farnesene biosynthesis. In both ‘Law Rome’ and ‘Idared’, a sharp increase in *AFS1* mRNA during the first 4–8 weeks of storage preceded a proportional rise in α -farnesene and a subsequent increase in CTols. However, maximum levels of *AFS1* transcript, α -farnesene, and CTols were, respectively, 2.5-, 4-, and 33-fold greater in ‘Law Rome’ than in ‘Idared’ apples. 1-MCP suppressed the increases in *AFS1* transcript and α -farnesene early in storage, although *AFS1* expression and α -farnesene synthesis had recovered in treated ‘Law Rome’ fruit after 20 weeks, consistent with increasing IEC in these fruit.

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1. Introduction

Superficial scald, a physiological storage disorder of apples and pears which manifests as brown or black patches on the fruit skin, results from necrosis

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of the hypodermal cortical tissue (Bain and Mercer, 1963) and is thought to be induced or exacerbated by oxidation products of the sesquiterpene (*E,E*)- α -farnesene (Huelin and Coggiola, 1970a, 1970b; Anet, 1972; Whitaker et al., 2000; Rowan et al., 2001). Fruit of scald-susceptible apple cultivars, such as ‘Granny Smith’, ‘Law Rome’, and ‘Delicious’ typically exhibit a relatively high rate of α -farnesene synthesis shortly after they are placed in low-temperature storage, which results in a marked accumulation of the sesquiterpene in the skin and epicuticular coating during the first 8–12 weeks (Huelin and Coggiola, 1968; Anet, 1972; Whitaker et al., 1997, 1998). The concentration of α -farnesene subsequently plateaus and then declines as its conjugated triene oxidation products increase to a maximum at about 16–24 weeks (Huelin and Coggiola, 1970a; Anet, 1972; Whitaker et al., 1997, 1998).

The primary in vivo oxidation products of α -farnesene that accumulate in apple epicuticular wax and peel tissue during cold storage have been identified as conjugated trienols (CTols), 9*E* and 9*Z* isomers of 2,6,10-trimethyldodeca-2,7,9,11-tetraen-6-ol (Rowan et al., 1995; Whitaker et al., 1997). Application of these CTols and their corresponding hydroperoxides to apple fruit prior to storage induced symptoms indistinguishable from naturally-occurring superficial scald (Rowan et al., 2001). Pre-storage treatment of apples with the antioxidant diphenylamine (DPA) inhibits oxidation of α -farnesene and largely prevents development of scald (Huelin and Coggiola, 1970a). Moreover, exposure of apple fruit to the blockers of ethylene action diazocyclopentadiene and 1-methylcyclopropene (1-MCP) greatly curtails α -farnesene production and markedly reduces scald incidence and severity (Gong and Tian, 1998; Fan et al., 1999; Rupasinghe et al., 2000a; Watkins et al., 2000; Shaham et al., 2003). Results from the studies with 1-MCP, as well as several previous reports (Watkins et al., 1993, 1995; Du and Bramlage, 1994; Whitaker and Solomos, 1997; Ju and Curry, 2000a), have shown that ethylene production and perception, and tissue responsiveness to ethylene, are involved in regulation of α -farnesene synthesis and induction of scald in apple fruit.

Studies utilizing radiolabeled precursors (Rupasinghe et al., 2001) or a statin inhibitor of 3-hydroxy-3-methylglutaryl-Coenzyme A reductase (HMGCR) (Ju and Curry, 2000b) have shown that in apple peel tissue α -farnesene is synthesized almost exclu-

sively via the mevalonic acid pathway, rather than the chloroplastic deoxyxylulose phosphate pathway. The final, rate-limiting enzyme in the pathway is α -farnesene synthase (FS), which converts farnesyl diphosphate (FDP) to α -farnesene (Rupasinghe et al., 1998, 2000b). We recently cloned and expressed the gene encoding FS from peel tissue of scald-susceptible ‘Law Rome’ apple fruit (*AFS1*; GenBank accession number AY182241), and moreover, we determined that an ethylene-dependent four-fold increase in *AFS1* transcript occurred during the initial 4 weeks of storage in air at 0.5 °C (Pechous and Whitaker, 2004). In this investigation, we cloned the corresponding *AFS1* gene from fruit of the scald-resistant cultivar ‘Idared’ (accession number AY523409) and compared *AFS1* expression during storage of ‘Law Rome’ and ‘Idared’ apples with respect to ethylene regulation, and in relation to the accumulation of α -farnesene and CTols and the post-storage incidence of superficial scald.

2. Materials and methods

2.1. Plant material, fruit treatment and storage, and tissue sampling

‘Law Rome’ and ‘Idared’ apple (*Malus domestica* Borkh.) fruit were harvested at commercial maturity from the Cornell University orchard at Lansing, NY. On the day of harvest, fruit were randomly allocated to treatments. Replicate samples of 10 fruit were used for measurement of internal ethylene concentration (IEC), and then peeled to obtain the tissue samples at harvest, which were immediately frozen in liquid N₂. Three replicate boxes of 100 fruit were treated with 1 μ l l⁻¹ 1-MCP (SmartFresh™, Agrofresh, Inc., Spring House, PA) in separate plastic containers as described by Watkins et al. (2000). Nontreated control fruit were sealed in identical containers without 1-MCP for the same duration. All fruit were subsequently stored in air at 0.5 °C and ~65% relative humidity. Nontreated fruit were sampled at 4, 8, 12, and 20 weeks, whereas 1-MCP-treated fruit were sampled at 4 and 20 weeks. At each sampling time, 10 fruit were removed from each treatment replicate, IEC was measured, the fruit were peeled around the circumference, and the tissue was immediately frozen in liquid N₂. Each 10-

fruit sample was processed individually immediately after removal from storage, while the fruit were still cold. To determine IEC, a 1 ml gas sample was withdrawn from the core of each apple using a 1 ml plastic syringe and analyzed by gas chromatography with flame ionization detection using a Hewlett-Packard 5890 Series II gas chromatograph (Agilent Technologies, Avondale, PA) fitted with a stainless steel column (2 m length \times 2 mm i.d.) packed with 60/80 mesh alumina F-1. Peel tissue samples were stored at -80°C until used for extraction of RNA or extraction and analysis of α -farnesene and CTols. The remaining fruit were removed from storage at week 20 and kept at 20°C for 1 week prior to visual assessment of scald incidence.

2.2. Extraction and quantification of α -farnesene and conjugated trienols (CTols)

Hexane extraction of α -farnesene and CTols from each replicate of frozen apple peel tissue was conducted as described in Whitaker et al. (1997) with the exception that 5 g tissue samples were immersed in 10 ml of hexane. Extractions were performed in triplicate. A 1 ml aliquot of each 10 ml extract was transferred to a 2 ml glass vial, the hexane was evaporated with a gentle stream of N_2 , and the residue was dissolved in 400 μl of methanol. The methanolic samples were analyzed by high-performance liquid chromatography (HPLC) using a Hewlett-Packard Series 1100 HPLC system (Agilent Technologies) with a quaternary pump, autosampler, and photodiode array detector (PDA). Sample vials were placed in the autosampler and 25 μl aliquots were injected onto a Luna 5 μm particle size C18(2) column (250 mm long, 4.60 mm i.d.) from Phenomenex (Torrence, CA) and eluted with isocratic methanol:water:acetonitrile, 90:5:5 (v/v/v), at a flow rate of 0.8 ml min^{-1} . PDA monitoring at 232 and 269 nm was used to determine levels of α -farnesene and CTols, which eluted at 13.8 and 5.8 min, respectively. HPLC-purified α -farnesene and CTol samples were used as external standards for quantification (Whitaker et al., 1997). Concentrations of these standards were calculated using the molar extinction coefficients $\varepsilon_{232\text{ nm}} = 27,740$ for α -farnesene (Huelin and Coggiola, 1968) and $\varepsilon_{269\text{ nm}} = 42,500$ for CTols (Anet, 1969).

2.3. Extraction and purification of RNA

Frozen apple peel samples (0.2 g) from each replicate were ground in liquid N_2 to a powder using a mortar and pestle and transferred to 15 ml screw-cap centrifuge tubes, followed by immediate addition of 2 ml of lysis buffer from the RNeasyTM RNA extraction kit (Ambion, Inc., Austin, TX). After vortexing and incubation for 10 min at 25°C , the samples were processed according to the kit protocol. The resulting RNA preparations were treated with DNase (TURBO DNase-freeTM from Ambion, Inc.) to remove genomic DNA, extracted with phenol:chloroform, 1:1 (v/v), and total RNA was precipitated by addition of one volume isopropyl alcohol plus 0.1 volume of 3 M sodium acetate, pH 5.5. The precipitated RNA was pelleted by centrifugation, washed with cold 70% ethanol, and resuspended in diethyl pyrocarbonate-treated water.

2.4. PCR amplification and cloning of 'Idared' AFS1 cDNA

Primers designed to amplify the coding region of 'Law Rome' AFS1 for in-frame cloning into the pET 3d expression vector (Pechous and Whitaker, 2004) were used to amplify and isolate the corresponding 'Idared' AFS1 cDNA. The forward primer, with the sequence 5'-ATATACCATGGAATTCAGAGTTCA-3', had the *Nco*I site of the vector preceding the ATG start codon for AFS1. The reverse primer, with the sequence 5'-GCCGGATCCTTAGTTTACAAGAGGTT-3', included a *Bam*HI restriction site after the stop codon. PCR was performed using 1 μl of a random-primed cDNA population from 'Idared' peel tissue mRNA as template, and was run for 35 cycles with a 55°C annealing temperature and an extension for 2 min at 72°C . The Invitrogen ElongaseTM PCR system (Invitrogen Life Technologies, Carlsbad, CA) was used to ensure full-length amplification of the 'Idared' AFS1 cDNA.

The PCR products were digested to completion with *Nco*I and *Bam*HI, and ligated overnight into the similarly digested pET 3d expression vector. The resulting recombinant plasmids were transformed into BL-21 DE3 (RIL) chemically competent *Escherichia coli* cells, and individual colonies were selected for expression and sequencing analyses. A sesquiterpene synthase assay of the soluble protein produced by expres-

sion of the full-length cDNA in transformed *E. coli* was performed as described in Pechous and Whitaker (2004).

2.5. Quantitative reverse transcription-polymerase chain reaction (RT-PCR)

An amount of 2 µg of total RNA per sample was used in the Thermoscript™ RT-PCR system with random priming (Invitrogen) to generate cDNA. Quantitative RT-PCR was performed using the QuantumRNA™ Universal 18S PCR mix (Ambion, Inc.) with a 9:1 competitor to 18S primer pair ratio. The ‘Law Rome’ *AFSI* gene-specific primers yielded a 461 bp PCR product, which included 92 bp of the cDNA 3′ untranslated region (UTR) and 369 bp of the 3′ end of the open reading frame (ORF). The *AFSI* reverse primer sequence was 5′-CAACAACGTTACAACTGTAAAG-3′ and the forward primer sequence was 5′-CACAA-GAATGAAGATCTTTTGTA-3′. The 18S universal primers yielded a 315 bp amplicon. The products of multiplex PCRs of each sample using both sets of primers were run on a 1.2% agarose gel and visualized by staining with ethidium bromide (0.1 µg ml⁻¹). The PCR products were quantified using a densitometry program in the Fluor-S™ imaging system (Bio-Rad Laboratories, Hercules, CA). The relative levels of *AFSI* primer products were then normalized based on the density of the 18S products for each sample.

3. Results

3.1. IEC and scald incidence

The mean IECs of ‘Law Rome’ and ‘Idared’ fruit at harvest were 3.0 and 1.8 µl l⁻¹, respectively. In ‘Law Rome’ control fruit, the mean concentration increased to 27.7 µl l⁻¹ by week 4, reached a maximum of 34.8 µl l⁻¹ by week 8, declined to 26.9 µl l⁻¹ by week 12, and declined further to 11.2 µl l⁻¹ at week 20. The mean IEC in 1-MCP-treated ‘Law Rome’ fruit was 1.1 µl l⁻¹ at week 4, but had increased to 9.8 µl l⁻¹ by the 20-week sampling time. In ‘Idared’ control fruit, the mean IECs were 7.6, 5.0, 7.9 and 8.3 µl l⁻¹ at 4, 8, 12 and 20 weeks, respectively. Mean IECs in 1-MCP-

treated ‘Idared’ fruit were 1.2 and 1.1 µl l⁻¹ at weeks 4 and 20, respectively.

The incidence of scald in untreated ‘Law Rome’ apples after 20 weeks at 0.5 °C plus 1 week at 20 °C was 86 ± 5%, whereas 1-MCP treatment prior to storage reduced the incidence to less than 1%. Fruit of ‘Idared’ showed no scald with or without 1-MCP treatment.

3.2. Accumulation of α-farnesene and CTols in apple peel tissue during storage

The concentration of α-farnesene in peel tissue of nontreated ‘Law Rome’ apples increased dramatically during the initial 4 weeks of storage, and then continued to increase linearly through 12 weeks, reaching a maximum of about 160 µg g⁻¹ and declining gradually thereafter (Fig. 1a). Accumulation of α-farnesene in peel tissue of nontreated ‘Idared’ apples occurred at a much slower rate and at 8–12 weeks the concentration plateaued at about 40 µg g⁻¹, four-fold lower than the maximum level in ‘Law Rome’. Levels of α-farnesene were very low in 1-MCP-treated fruit of both cultivars after 4 weeks of storage, but after 20 weeks the α-farnesene concentration in ‘Law Rome’ was substantial (80 µg g⁻¹) and more than four-fold greater than that in ‘Idared’.

CTols in peel tissue of nontreated ‘Law Rome’ fruit increased very gradually during the first 8 weeks of storage and then very rapidly from 8 to 12 weeks, reaching a maximum concentration of about 30 µg g⁻¹ that was maintained until the end of storage at 20 weeks (Fig. 1b). In marked contrast, there was very little accumulation of CTols throughout the storage of nontreated ‘Idared’ fruit, and the maximum concentration of 0.9 µg g⁻¹ at 12 weeks was about 33-fold lower than that in ‘Law Rome’. Pre-storage 1-MCP treatment of ‘Law Rome’ apples greatly attenuated the accumulation of CTols, but low-levels of CTols were present at 20 weeks, when recovery of α-farnesene production was evident.

3.3. Nucleotide and encoded amino acid sequences of ‘Law Rome’ and ‘Idared’ *AFSI*

Sequence analysis determined that the coding region of the ‘Idared’ *AFSI* cDNA (GenBank accession

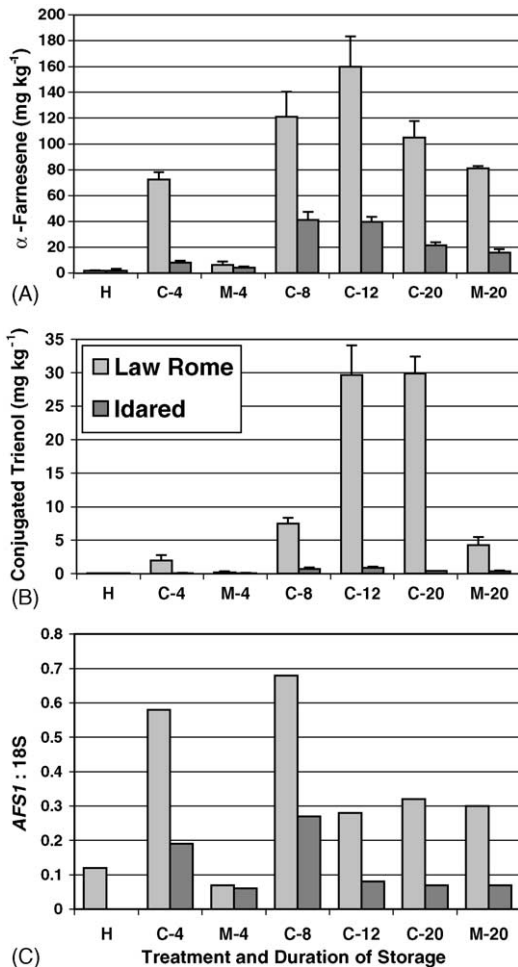


Fig. 1. Concentrations of α-farnesene (a), and its conjugated trienol (CTol) oxidation products (b), and *AFS1* transcript levels (c), in peel tissue of nontreated control and 1-MCP-treated 'Law Rome' and 'Idared' apple fruit stored at 0.5 °C in air for 0–20 weeks. Fruit sampled at harvest, control fruit sampled at 4, 8, 12, and 20 weeks, and 1-MCP-treated fruit sampled at 4 and 20 weeks, are designated as H, C-4, C-8, C-12, C-20, M-4, and M-20, respectively. Separation and quantification of α-farnesene and CTols were performed by C₁₈-HPLC with UV monitoring at 232 and 269 nm, using HPLC-purified α-farnesene and CTol samples of known concentration as external standards. The Ambion QuantumRNA™ quantitative RT-PCR kit was used to determine levels of *AFS1* cDNA relative to the control 18S cDNA. Ethidium bromide-stained gels of the RT-PCR products were used for densitometric measurements of the amplified *AFS1* and 18S cDNA fragments, and the graph bars show the *AFS1*:18S gel band density ratio. Error bars in (a) and (b) represent the standard deviation of the mean ($n=3$).

number AY523409) is more than 99% identical to that of the *AFS1* cDNA from 'Law Rome' (AY182241), with only seven nucleotide substitutions in the 1728 bp ORF. The encoded amino acid sequences of the two *AFS1* genes (GenBank accession numbers AAS01424 and AAO22848) were found to differ by only five of 576 amino acids, with two of the pairs of non-identical amino acids being chemically similar. Two domains critical to enzymatic function, the DDXXD divalent cation-binding domain (amino acids 326–330), common to all terpene synthases, and the RR(X₈)W motif near the N-terminus (amino acids 33–43), common to the *Tps-b* and *Tps-d* gene families (Dudareva et al., 2003), are identical in the 'Idared' and 'Law Rome' *AFS1* encoded proteins. The soluble protein produced by expression of the full-length 'Idared' *AFS1* cDNA in transformed *E. coli* yielded >99% (*E,E*)-α-farnesene in a sesquiterpene synthase assay (data not shown), as was determined previously for bacterially expressed 'Law Rome' *AFS1* (Pechous and Whitaker, 2004).

3.4. *AFS1* transcript levels in control and 1-MCP-treated fruit

In peel tissue of fruit of both cultivars, expression of the α-farnesene synthase gene *AFS1* increased during the first 4 weeks of storage, peaked at 8 weeks, and was strongly inhibited by pre-storage treatment with 1-MCP (Fig. 1c). Low-level expression was evident in 'Law Rome' fruit at harvest, whereas *AFS1* transcript was not detected in newly harvested 'Idared' fruit. Over the 20 weeks of storage in air at 0.5 °C, *AFS1* mRNA was consistently more abundant (about 2.5- to 4-fold higher) in 'Law Rome' than in 'Idared' control fruit. 1-MCP-treated fruit of the two cultivars showed a similar low-level of *AFS1* expression after the initial 4 weeks of storage, whereas after 20 weeks transcript had increased about four-fold in 'Law Rome' but only slightly in 'Idared'.

The transient rise in the abundance of *AFS1* transcript in nontreated 'Law Rome' apples was plotted in relation to changing peel tissue concentrations of α-farnesene and CTols over the course of storage (Fig. 2). An early increase in *AFS1* mRNA preceded a similar increase in α-farnesene; *AFS1* transcript peaked at about 8 weeks and then declined, whereas α-farnesene peaked at 12 weeks and then declined. Accumulation of CTol oxidation products of α-farnesene began at about

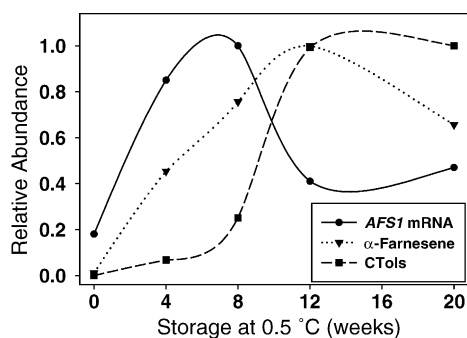


Fig. 2. Changes in the relative levels of *AFS1* mRNA, α -farnesene, and conjugated trienols (CTols) in peel tissue of nontreated control 'Law Rome' apple fruit over the course of 20 weeks of storage at 0.5 °C in air. Data points were normalized relative to the highest value determined for each constituent, which was assigned a value of 1.0.

7 weeks and plateaued at about 12 weeks, coincident with the decline in α -farnesene.

4. Discussion

For about 40 years, the apple industry has controlled superficial scald by pre-storage treatment of susceptible fruit with the antioxidant DPA, which retards the autoxidation of α -farnesene (Huelin and Coggiola, 1968, 1970; Anet and Coggiola, 1974) and appears to have other beneficial effects on fruit physiology, including reduced rates of respiration and ethylene evolution (Lurie et al., 1989; Du and Bramlage, 1994; Whitaker, 2000, 2004). A negative aspect of the DPA drench treatment is that a fungicide must be included to limit postharvest decay. Hence, this practice results in unwanted chemical residues on the fruit, which has prompted the search for alternative methods of scald control. A promising recent development in this effort was the demonstration that pre-storage treatment of apples with 1-MCP, a blocker of ethylene receptor sites, greatly diminished α -farnesene production and largely prevented scald (Fan et al., 1999; Rupasinghe et al., 2000a; Watkins et al., 2000; Shaham et al., 2003). These results showed a direct link between ethylene perception and the burst of α -farnesene synthesis shortly after apples are placed in low-temperature storage. This raised the question of how ethylene stimulates production of the sesquiterpene.

Our laboratory and others have recently explored the possibility that ethylene regulates transcription and translation of genes in the α -farnesene biosynthetic pathway. Complete or partial cDNAs have been cloned that encode three HMGR isozymes, a farnesyl diphosphate synthase, and an (*E,E*)- α -farnesene synthase (Whitaker, 2004). Of these genes, the ethylene-dependent upregulation of 'Delicious' *HMG2* (GenBank accession number AF316112; Rupasinghe et al., 2001) and 'Law Rome' *AFS1* (AY182241; Pechous and Whitaker, 2004) in the first several weeks of storage is consistent with a key role in the marked increase in α -farnesene synthesis. In the present study, we cloned the *AFS1* gene (AY523409) from fruit of 'Idared', a scald-resistant cultivar, to enable comparison of its expression with that of *AFS1* in fruit of scald-susceptible 'Law Rome'. The results showed that although the pattern of ethylene-regulated *AFS1* expression was quite similar in fruit of the two cultivars, the level of *AFS1* transcript was always substantially higher in 'Law Rome' (Fig. 1c). It was also observed that suppression of *AFS1* expression in 1-MCP-treated 'Law Rome' apples was at least partially overcome after 20 weeks of storage. The late rise in *AFS1* transcript coincided with increased IEC in these fruit, suggesting recovery of ethylene responsiveness.

The temporal pattern and level of α -farnesene accumulation during storage paralleled the expression of *AFS1* in both 'Law Rome' and 'Idared' fruit (Figs. 1a, c, and 2). It can be inferred from this that translation of *AFS1* mRNA and consequent FS activity were proportional to the abundance of *AFS1* mRNA, although other pathway enzymes and the levels of substrates may also influence the rate of α -farnesene production (Rupasinghe et al., 2001). It is unlikely that the FS products of the 'Law Rome' and 'Idared' *AFS1* genes differ in their activity or kinetics, in as much as the amino acid sequences of the two enzymes are more than 99% identical and do not differ in either the DDXXD or the RR(X)₈W motif. Generation of FS-specific antibodies and immunoblot analysis of apple peel tissue proteins will be required to evaluate translation versus transcription of *AFS1*.

The maximum concentration of α -farnesene was about four-fold higher in 'Law Rome' than in 'Idared' apples, whereas the maximum concentration of CTols was about 33-fold higher in 'Law Rome' (Fig. 1). Thus, the proportion of α -farnesene that undergoes autoxidation

tion during storage appears to be much greater in fruit of the scald-susceptible cultivar. Autoxidation of α -farnesene is thought to involve a free radical-mediated reaction, which increases dramatically once a threshold level of oxidized α -farnesene accumulates (Anet, 1974). The relatively high level of CTol accumulation in peel tissue of 'Law Rome' fruit might result from rapid depletion of natural antioxidants early in storage (Anet and Coggiola, 1974; Whitaker, 2004) and/or much lower peroxidase activity in 'Law Rome' compared with 'Idared' peel tissue during storage (Fernández-Trujillo et al., 2003). The CTols found in stored apple fruit are a reduced form of the corresponding conjugated triene hydroperoxide isomers produced during α -farnesene autoxidation (Anet, 1969; Rowan et al., 1995), and it is currently not known how this reduction to the alcohols occurs. Possibly it involves an enzyme, in which case activity of the 'reductase' could be another factor controlling CTol accumulation in fruit of different apple cultivars (Whitaker, 2004).

Previous studies have shown a correlation between levels of conjugated triene oxidation products of α -farnesene in cold-stored apples and scald severity after removal of the fruit from storage (Huelin and Coggiola, 1970b; Anet and Coggiola, 1974; Watkins et al., 1993; Whitaker and Solomos, 1997; Rowan et al., 2001). Accumulation of a high concentration of α -farnesene is relatively poorly correlated with subsequent development of severe scald, indicating that the onset and rate of α -farnesene oxidation are critical (Anet, 1972, 1974; Anet and Coggiola, 1974). Nevertheless, rapid α -farnesene synthesis early in storage typically results in the relatively early accumulation of high levels of CTols in fruit of scald-susceptible cultivars (Whitaker and Solomos, 1997; Whitaker et al., 1998; Watkins et al., 2000). On the basis of this observation and the results of this study showing a close correlation of *AFSI* transcript abundance with α -farnesene production (Fig. 2), we conclude that molecular genetic manipulation to suppress *AFSI* expression in stored fruit of scald-susceptible cultivars such as 'Law Rome' is likely to largely prevent scald development.

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